Journal of Medical Virology 57:390–396 (1999)

Viral Diarrhea in Children in Beijing, China

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A study was undertaken from November 1994 to August 1996 to determine the role of viruses in children (≤5 years of age) hospitalized at Beijing Children Hospital, Beijing China, for acute diarrhea. Stool samples from diarrheal patients were investigated by ELISA, electron microscopy, and RT-PCR for the presence of rotavirus, calicivirus, astrovirus, and adenovirus. Group A rotavirus was detected in 55.9% of all diarrheal patients and comprised 82.5% of all viruses detected. Group A rotavirus samples were further characterized for their G-type specificity by RT-PCR. Four major G types (1–4) were identified. G1 to G4 accounted for 58.9%, 15.7%, 16.8%, and 6.3%, respectively, of the serotyped samples. Almost all rotavirus infections occurred in children less than 1 year of age, with a significant clustering during the winter months. Group C rotavirus was detected in one 18-month-old child. Astroviruses, caliciviruses, and adenoviruses were detected in 8.5%, 7.6%, and 2.5% of the hospitalized children, respectively. This, the first viral etiological study of childhood diarrhea in China, concludes that rotavirus G1–4 strains play an important role in severe diarrhea in Beijing children. ***J. Med. Virol. 57:390–396, 1999.***

KEY WORDS: rotavirus; gastroenteritis, calicivirus; astrovirus; adenovirus

INTRODUCTION

During the past 2 decades viruses have been firmly established as etiological agents of acute gastroenteritis [Bern and Glass, 1994]. Four major categories of viruses are now recognized as clinically important including rotavirus, astrovirus, adenovirus, and calicivirus [Bern and Glass, 1994]. Among these four viruses, rotavirus is the single most important etiologic agent of serious diarrhea in young children and responsible for millions of cases of severe diarrhea and 870,000 deaths per year [Cook et al., 1990; Bern and Glass, 1994]. The development of an effective rotavirus vaccine to reduce mortality in developing countries and morbidity in developed countries has therefore become an important priority [Glass et al., 1997; Vesikari, 1997].

Unfortunately, clinical trials with several rotavirus vaccine candidates of bovine and rhesus monkey origin have shown different protective efficacies [Kapikian, 1994; Vesikari, 1994; Glass et al., 1996]. The reasons are not recognized, but it is generally thought that the presence of multiple serotypes in a vaccine would favor the development of a broadly protective immune response. In order to optimize a vaccine, it is therefore important to gain information about the circulating rotavirus serotypes in a community [Gentsch et al., 1996].

In a large developing country such as China, introduction of a potent rotavirus vaccine would most likely be beneficial for children. However, before a rotavirus vaccine can be introduced or evaluated, epidemiological and clinical information about circulating rotavirus serotypes and the role of other intestinal viruses is required. So far, the epidemiological information about viruses as causes of infantile diarrhea in China is very limited. Furthermore, the epidemiological information about circulating rotavirus serotypes is scarce [Fang et al., 1994], and no information is available on the prevalence or the importance of caliciviruses, enteric adenoviruses, or astrovirus in Chinese children.

MATERIALS AND METHODS

Clinical Specimens

Fecal samples (1/child) were collected from November 1994 to August 1996 from 186 children ≤5 years of age with a clinical diagnosis of acute diarrhea admitted to Beijing Children Hospital. All specimens were stored at −70°C until use.

Grant Sponsor: Swedish Medical Research Council; Grant number: K98-o6X-10392-o6B; Grant Sponsor: Swedish Board for Investment and Technical Support (BITS).

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Accepted 7 October 1998

Rotavirus ELISA

All fecal samples were initially screened for group A rotavirus in China by a previously described enzyme-linked immunosorbent assay [Espinoza et al., 1997].

Electron Microscopy (EM)

Briefly, one drop of a 10% stool suspension was incubated on Formvar/carbon-coated copper grids (400 mesh) for 1 min and then stained with 2% phosphotungstic acid (pH 6.0) followed by examination in a Philips CM 100 electron microscope at a magnification of 46,000×. The sample was considered negative if virus was not detected after 10 min of examination.

RT-PCR for Rotavirus Serotyping

Rotavirus double-stranded RNA was extracted and purified by guanidinium thiocyanate (GTC) and silica, essentially as described by Boom et al. [1990].

Serotyping of rotavirus strains by PCR was performed as described by Gouvea et al. [1990] with minor modifications. Briefly, 10 μl of purified rotavirus dsRNA was mixed with 0.8 μl of methyl mercuric hydroxide (100 mM) and 3 μl of a mixture of A2 primers (End 9, End 9-UK, 33 μM each) [Gouvea et al., 1990], after 5 min of incubation at room temperature, 0.8 μl of beta-mercaptoethanol (700 mM) was added and incubated for another 5 min at room temperature. The denatured template was then mixed with a reverse transcriptase (RT) mixture containing 3 μl of 10× PCR buffer (Perkin-Elmer preformulated buffer, Perkin-Elmer, Norwalk, CT), 1.8-μl $MgCl_2$ (Perkin-Elmer buffer, 25 mM), 3-μl dNTPs (Pharmacia Biotech, 2 mM/each), 6.6 μl of H_2O, 0.5 μl (100 U) of reverse transcriptase (Superscript, GIBCO, Bethesda, MD), 0.5 μl (20 U) of RNAse inhibitor (Promega, Madison, I). After mixing, the tubes were placed in the thermal cycle for 60 min at 42C° for complementary DNA synthesis. The DNA amplification and G-typing were performed as described by Gouvea et al. [1990] using serotype-specific primers (*a*BT1, *a*CT2, *a*ET3 and *a*DT4; or *a*AT8, and *a*FT9).

RT-PCR for Detection of Group C Rotavirus

RNA extraction and RT-PCR were performed essentially as described above. Briefly, RNA was denatured in 3-mM methyl mercuric hydroxide and added to a reaction mix composed of 10-mM Tris (pH 8.3) 50-mM KCl, 1.5-mM $MgCl_2$, 0.2-mM dNTP, 20-U RNasin (Promega), 100-pmol 3′ end primer GCAACAGTGTAGTTGGATAG (bp 1129–1148, gene 5, Cowden strain) and 100-U M-MLV RT (Superscript GIBCO). After incubation at 42°C for 1 hr, enzyme was inactivated at 95°C for 5 min, followed by the addition of a PCR mix composed of 10-mM Tris (pH 8.3), 50-mM KCl, 0.2-mM dNTP, 1.5-mM $MgCl_2$, 100-pmol 5′ end primer ATTGAAGCTGGTGCTCCA (bp 693–711, gene 5, Cowden strain) and 2.5-U Taq polymerase. Thirty amplification cycles were carried out (2 min at 94°C, 1 min at 50°C, and 1 min at 72°C, followed by a final incubation at 72°C for 5 min.

RT-PCR for Calicivirus

Samples positive for calicivirus by EM were studied further by RT-PCR essentially as described by Guyader et al. [1996]. Viral RNA was extracted and purified from stools by the same methods as described above for rotavirus. The following primers were used: NVp 110 (–) 5′-AC(A/T/G)AT(C/T)TCATCATCACCATA-3′ (location 4865–4884); NVp 36 (+) 5′-ATAAAAGTTGGCATGAACA-3′ (location 4487–4501); NVp 69 (+) 5′-GGCCTGCCATCTGGATTGCC-3′ (location 4733–4752); NI (+) 5′-GAATTCCATCGCCCACTGGCT-3′ (location 4768–4788) [Le Guyader et al., 1996]. Briefly, 10 μl of purified calicivirus ssRNA was mixed with 1 μl of methyl mercuric hydroxide (100 mM) and 1 μl of a NVp 110 (33 uM), after 5 min of incubation at room temperature, 1 μl of beta-mercaptoethanol (700 mM) was added and incubated for another 5 min at room temperature. The mixture was subsequently added to an RT reaction cocktail containing 10-mM Tris, 50-mM KCl, 5-mM $MgCl_2$, 0.5 mM each dNTP, 20 U of RNAsin, and 100 U of Superscript, and incubated for 1 hr at 42°C, followed by 95°C for 5 min. The RT reaction (30 μl) was mixed with 70 μl of PCR reaction mixture containing 10-mM Tris-HCl, 50-mM KCl, 2-mM $MgCl_2$,1 μM each of primer NI, NVp 69, and NVp 36, 0.3-mM of each dNTP, and 2.5 U of Taq Polymerase. The amplification cycle program included denaturing at 94°C for 4 min, 40 amplification cycles (denaturation at 94°C for 1 min, annealing at 45°C for 1 min, extension at 72°C for 1 min), followed by a final incubation at 72°C for 10 min. The PCR products were analyzed on 2% agarose gels as described above.

RNA Gel Electrophoresis and Silver Staining

Rotavirus RNA was extracted, separated by PAGE, and stained with silver nitrate [Svensson et al., 1986].

RESULTS

Viruses Detected in Stools From Children With Acute Gastroenteritis

Stool samples from 186 children with acute gastroenteritis collected at Beijing Children Hospital from November 1994 to August 1996 were screened for group A rotavirus by ELISA in China. Due to the limited amount of material, only 118 of these 186 samples were further examined in Stockholm for other viruses. Samples that were rotavirus-positive by ELISA in Beijing were also reexamined in Stockholm for group A rotavirus; no false positives were identified (data not shown). Group A rotavirus was detected by ELISA in the stools of 104/186 children (55.9%) and astrovirus in 10/118 children (8.5%) by EM (Table I). Of the 10 astrovirus-infected children, 3 were also infected with rotavirus. Interestingly, adenoviruses were only found in the stools of three (2.5%) children by EM (Table I) and

TABLE I. Enteric Viruses Detected in Children With Acute Gastroenteritis in Beijing

	1994 (November–December)	1995 (January–December)	1996 (January–August)	Total (%)
Patients	42	86	58	186
GRP-A[a]	34	48	22	104/186 (55.9)[c]
GRP-C[b]	0	0	1	1/118 (0.8)
Astrovirus	2	4	4	10/118 (8.5)
Adenovirus	0	1	2	3/118 (2.5)
Calicivirus	1	3	5	9/118 (7.6)

[a]Group A rotavirus ELISA.
[b]Group C rotavirus PCR.
[c]Percent within paranthesis.

in all these cases as dual infections together with rotavirus or astrovirus.

Human calicivirus (HuCV) was found in 9 of the 118 stools (7.6%) by EM and/or RT-PCR (Table I). Figure 1 illustrates detection of HuCV by RT-PCR. Of the nine EM positive samples, four were positive using the primer pair NVp 110–NI, and three other samples were positive with primer pair NVp 110–NVp69. Two of the EM-positive samples were negative and none of the nine EM-positive samples were amplified with the primers NVp 110–NVp 36.

The rationale to combine EM and group A rotavirus ELISA was to search for non–group A rotaviruses. In only a single stool did we find Rotavirus particles by EM which was repeatedly negative by group A ELISA and group A PCR. RNA gel electrophoresis indicated a group C RNA profile [von Bonsdorff and Svensson, 1988; Maunula et al., 1992], but as the RNA profile was too faint to be documented after silver staining (data not shown), a group C-specific PCR was established based on gene 5 encoding vp6 of the Cowden strain. PCR amplification established that the isolate belonged to group C rotavirus (Fig. 2).

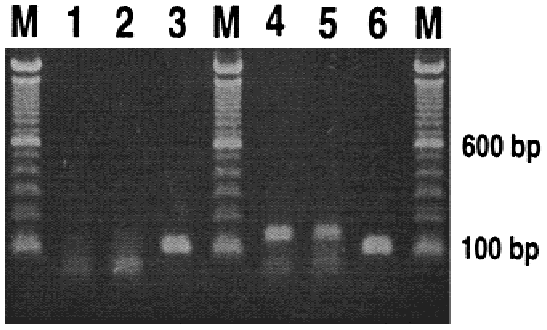

Fig. 1. Detection of human Calicivirus by RT-PCR. Lanes marked M indicates size marker (100-bp DNA ladder). Lanes 1–2 shows RT-PCR–negative samples; lanes 3 and 6 showing HuCV amplified with primers Nvp 110–NI (114 bp); lanes 4 and 5 HuCV amplified with primers Nvp 110–Nvp 69 (151 bp).

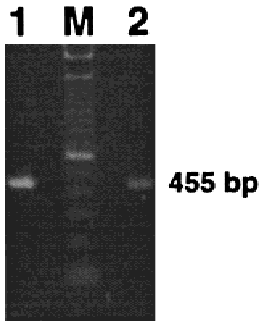

Fig. 2. Detection of group C rotavirus by RT-PCR and ethidium bromide staining. Lane 1 shows positive control of porcine group C rotavirus (strain Cowden); lane M, size marker 100-bp DNA ladder; lane 2, fecal sample RN-77.

Seasonal Distribution of Viral Intestinal Pathogens

The frequency of different viruses found per month during the study period is presented in Figure 3. Rotaviruses were detected during virtually all winter months except in January 1995, when no specimens were collected. The highest prevalence of rotavirus was observed in November and December, in both years accounting for 63.4% of all 104 positives found. Astroviruses demonstrated a seasonal pattern similar to rotavirus, with a predominance during the winter months. Unlike rotavirus and astrovirus, the few HuCV cases that were identified occurred scattered throughout the study period. A seasonal pattern for adenovirus was not possible to establish due to the limited number of cases detected.

Sex and Age Distribution

Of the 186 children that visited the hospital, 31 were girls (16.7%). The age distribution of the infected children is presented in Table II. It should be noted that age information, due to registration procedures, was available for only 74 of the 104 children infected with rotavirus. The most surprising observation was that more than 75% of the infections for each virus occurred in children within the first year of life. In fact, all of the infections caused by astrovirus and adenovirus occurred within the first year of life (Table II). Table II also shows that practically all symptomatic rotavirus infections occurred before 2 years of life. The single case of group C rotavirus infection occurred in a 18-month-old boy.

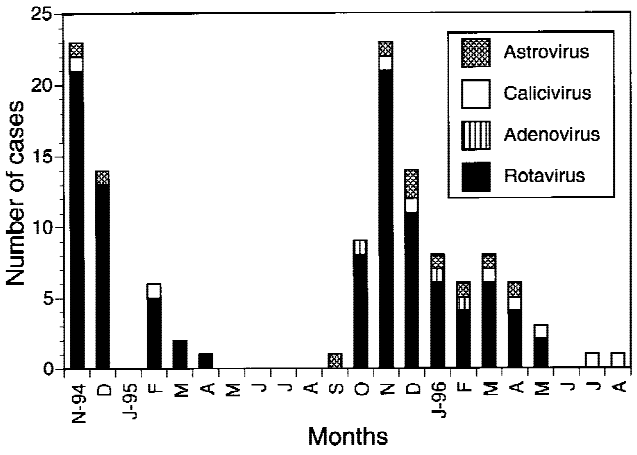

Fig. 3. Seasonal distribution of viral intestinal pathogens in children admitted to Beijing Children Hospital with acute diarrhea during November 1994–August 1996.

Seasonal Distribution of Rotavirus Serotypes

Using a single-amplification PCR procedure including methyl mercuric hydroxide for efficient RNA denaturation during RT reaction, we succeeded to G-type 95 out of 114 (83%) rotavirus samples with primers representing serotypes 1–4, 8, and 9. The molecular weights of the PCR products and serotype specificities were identical to those previously described by Gouvea et al. [1990]. Table III shows that serotype 1–4 strains cocirculated during the study period, with serotype 1 being the dominating serotype. Table III also shows that G2 and G3 strains were about equal in frequency. The monthly occurrence of each serotype from November 1994 to August 1996 is presented in Figure 4 and shows that G1 strains dominated all months except March–April 1995 and April–May 1996. G2 strains disappeared in February 1995 and reoccurred intermittently from October 1995 to May 1996. Serotype 3 strains were common throughout the study period.

Age Distribution and Rotavirus Serotypes

To investigate if a certain serotype was specifically associated with a certain age group, age and serotype data were compiled and are shown in Table IV. It is interesting to note that while G1 strains were predominating during the study, they were not detected in any child older than 24 months; in fact, only two cases of rotavirus infections were found in children older than 24 months (Table IV): one was a G8 strain found in a 5-year-old boy and the other was a G3 strain found in a 36-month-old boy.

DISCUSSION

This is to our knowledge the first study to document the role of viruses in childhood diarrhea in China. The survey that was performed during November 1994 to August 1996 in Beijing revealed that 55.9% of the diarrheal patients ($\leq$5 years of age) seeking hospital treatment were infected with rotavirus. Previous studies carried out in Latin America and other developing countries have also indicated that rotavirus is an important pathogen responsible for 7%–65% of all diarrhea episodes [Black et al., 1981; Mata et al., 1983a, 1983b; Lhinares et al., 1989; Kim et al., 1990; Raul Velazquez et al., 1993; Prado and O'Ryan, 1994]. Hospital admissions for rotavirus disease show fluctuations throughout the year, with most hospitalizations occurring during the winter season in temperate regions. Also, in this study a majority of the infections with rotavirus were observed during the cooler months.

It has previously been shown that the highest rate of rotavirus infections appears in children aged 6–18 months and that this group also shows the highest number of hospital admissions [Bishop, 1994]. A similar observation was made in this study: more than 50% of the infections occurred in children between 7 and 12 months of age, followed by the 0–6-month age group. In fact, a majority (97.3%) of rotavirus infections were seen in children younger than 2 years of age, supporting the belief that these infections occur early in life.

It was interesting to note that a majority of rotavirus infections occurred in boys. This sex ratio difference was without any apparent explanation. Due to incomplete clinical recordings, no attempts were made to investigate correlations between clinical severity and sex among the patients.

In this study, an assessment of the distribution of vp7 (G-type) serotypes was made. Using a slightly modified PCR method originally described by Gouvea et al. [1990], G-typing was successful in 83% of the rotavirus positive samples. While information about G-type distribution is available from several countries [Gentsch et al., 1996], there is as yet a limited amount of information from large developing countries such as China [Fang et al., 1994]. China is a developing country that most likely would benefit from rotavirus vaccination, but before a vaccine strategy can be chosen serotype information must be available for vaccine policy makers. Despite the relatively small numbers of specimens ($n = 95$), the results indicate that the worldwide predominating serotypes 1–4 [Gentsch et al., 1996] are also those that predominate in Beijing; all four major serotypes cocirculated during the study period, with G1 being the predominant serotype (58.9%). A similar predominance of serotype 1 strains has also been reported from other parts of China [Fang et al., 1994] as well as from other parts of the world [Gentsch et al., 1996]. In contrast to G4 strains, which were only sporadically detected, G2 and G3 strains were almost equal in distribution and numbers. Only a single G8 infection was identified. Interestingly, the patient was 5 years old, being thus the oldest child from whom G-typing information was available.

Apart from investigating the role of group A rotavirus in acute diarrhea, the intention was also to search for non–group A rotaviruses, i.e., groups B and C. Only one specimen was rotavirus-positive by EM and a group C-specific PCR, but negative by group A-specific ELISA and PCR. To our knowledge, this is the first

TABLE II. Age and Pathogen Distribution in Children With Acute Diarrhea[a]

	Age, months				
	0–6	7–12	13–24	>24	Total
Rotavirus	17 (23.0%)	40 (54.0%)	15 (20.3%)	2 (2.7%)	74
Calicivirus	6 (66.7%)	2 (22.2%)	1 (11.1%)		9
Astrovirus	8 (80%)	2 (20%)			10
Adenovirus	2 (67%)	1 (33%)			3

[a]Age information was only available for 74 of 104 group A rotavirus-positive children.

TABLE III. Rotavirus Serotypes in Children Admitted to Beijing Children Hospital

	Serotype (G-type)							Number of specimens serotyped
Year	1	2	3	4	8	9	1+3	
1994	18	4	5	3			1	31
1995	27	7	8	2	1			45
1996	11	4	3	1				19
Total	56	15	16	6	1	0	1	95

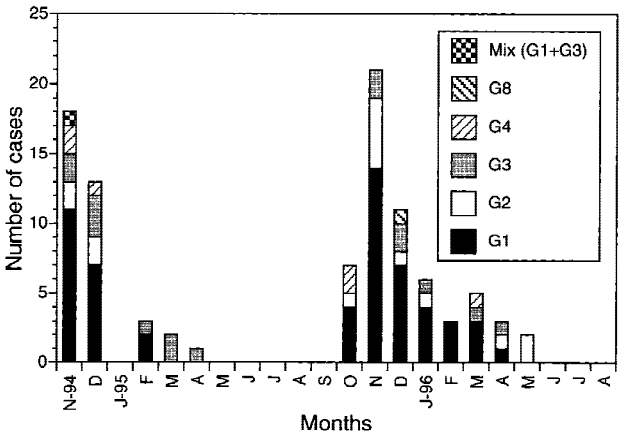

Fig. 4. Distribution of rotavirus serotypes in children admitted to Beijing Children Hospital with acute diarrhea during November 1994–August 1996.

group C isolate identified in China. Interestingly, this isolate was detected in a relatively young child (18 months), which is in contrast to most other group C isolates that have been recognized in older children and adults [von Bonsdorff and Svensson, 1988; Brown et al., 1989; Matsumoto et al., 1989; Maunula et al., 1992].

Recent characterization of HuCV has resulted in improved methods for detection as well as classification of these viruses. HuCV are presently classified into three (1–3) different genogroups [Liu et al., 1995; Estes et al., 1997], with genogroup 3 comprising viruses that possess "classical" calicivirus morphology [Cubitt, 1987; Liu et al., 1995; Estes et al., 1997]. A combination of EM and PCR with primers recognizing genogroups 1 and 2 strains [Le Guyader et al., 1996] allowed us to detect caliciviruses for the first time in Chinese patients. Four isolates were found to belong to genogroup 2 and three to genogroup 1. One of the two specimens that were negative by PCR had classical calicivirus morphology. In this study, 7.6% altogether of the hospitalized children were positive for calicivirus, with all infections occurring before 2 years of age. In a similar study from South Africa, Wolfaardt et al. [1997] detected caliciviruses in 3.3% of the patients, predominantly from children less than 4 years of age. Unlike the situation in Sweden, where genogroup 2 strains dominate (data not shown), genogroup 1 strains dominated in Beijing. However, the limited number of caliciviruses identified makes it premature to speculate on predominance of genogroups or epidemiology patterns.

Adenoviruses were found in 3/118 (2.5%) children with acute diarrhea. The fact that enteric adenovirus infections have a symptomatology that is milder than that of rotavirus may have limited the number of children with adenovirus infections to seeking hospital attention. In a 1-year prospective study from Sweden, Uhnoo et al. [1984] found enteric adenoviruses in 8% of children with acute diarrhea. A majority of these infections occurred before 2 years of age. Information concerning the occurrence of enteric adenoviruses in other regions is complex. In Brazil and Bangkok, 2% of children with diarrhea shed enteric adenoviruses, whereas in South Africa and Guatemala, 13% and 31%, respectively, of children with diarrhea excreted enteric adenoviruses [Leite et al., 1985; Herrmann et al., 1988; Tiemessen et al., 1989; Cruz et al., 1990; Wadell et al., 1994].

In this study, all adenovirus infections occurred before 1 year of age and all patients required hospitalization. However, as all episodes were dual infections, the role of enteric adenovirus in severe diarrhea could thus not be determined. In fact, the few isolates identified suggest that this pathogen play a minor role in severe childhood diarrhea in Beijing.

An interesting observation from this study was not only that as many as 8.5% of the examined children were infected with astrovirus, which makes it the second most common virus in this study, but more importantly that this relatively mild disease [Greenberg and Matsui, 1992] was so severe in these children (all $\leq$2 years of age) that they required hospitalization. It should be noted, however, that 3/10 children were also infected with rotavirus, so the severity of symptoms caused only by astrovirus in these cases could not be determined. Furthermore, the fact that bacterial pathogens were not examined makes it difficult to rule out the possibility that astrovirus was the only pathogen associated with symptoms. While the literature report outbreaks of astrovirus in daycare settings, schools, and nosocomial outbreaks [Esahli et al., 1991;

TABLE IV. Age Distribution of Rotavirus Serotypes in Children With Acute Diarrhea in Beijing, China, 1994–1996

	Number of rotavirus serotypes					
Age, months[a]	G1	G2	G3	G4	G8	G9
0–6	12 (27.9%)	2 (14.3%)	2 (16.7%)	1 (25%)		
7–12	22 (51.2%)	8 (57.1%)	7 (58.3%)	3 (75%)		
13–24	9 (20.9%)	4 (28.6%)	2 (16.7%)			
>24			1 (8.3%)[b]		1 (100%)[c]	
Total (#74)	43	14	12	4	1	0

[a]Note that the age was only known for 74 out of 95 positive by PCR.
[b]Patient was 36 months old.
[c]Patient was 5 years old.

Lew et al., 1991; Noel and Cubitt, 1994; Oishi et al., 1994; Palombo and Bishop, 1996; Shastri et al., 1998], there are few if any reports describing hospitalization due to severe symptoms caused by astrovirus. As we are not aware of previous outbreaks with astrovirus in China, it is too premature to speculate if astroviruses in China cause a more severe clinical picture than elsewhere. It is clear, however, that astroviruses are frequently found in childhood diarrhea in Beijing and that the clinical importance of astroviruses in acute diarrhea should be further examined in China.

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